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# **Transformation of TNT to Triaminotoluene by Mixed Cultures Incubated Under Methanogenic Conditions**

by

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2,4,6-Trinitrotoluene (TNT) is an explosive widely used by the military. Although it is no longer manufactured in the United States, large amounts of wastewater are generated annually from load, assembly, and packing (LAP), and demilitarization operations. Granular activated carbon adsorption is the standard technology for treating wastewater containing TNT and maintaining discharges within the limits established under the National Pollutant Discharge Elimination System. Previous studies evaluating biological treatment of pink water using anaerobic fluidized bed granular activated carbon bioreactors have been promising. Our objectives for this work were to study the end-products produced during the anaerobic biodegradation of TNT and study the effect of adding cosubstrates on TNT degradation.

These studies demonstrated TNT was initially reduced to a variety of reduction products that culminated in the formation of triaminotoluene (TAT). TAT was susceptible to further degradation under anaerobic conditions, but its fate was not determined. The addition of ethanol and glucose enhanced the degradation of TNT, but acetate did not. These studies demonstrate, for the first time, near stoichiometric formation of TAT in a mixed culture incubated under methanogenic conditions, and demonstrate the importance of adding reduced cosubstrates to enhance the formation of TAT.

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## Foreword

This study was conducted for Headquarters, U.S. Army Corps of Engineers (HQUSACE), under Project 4A161102AH68, "Processes in Pollution Abatement Technology"; Work Unit EG7, "Pathways and Controlling Factors in Biodegradation of Energetic Wastes." The technical monitor was Dr. Stephen W. Maloney, CECER-UL-I.

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# 1 Introduction

## Background

2,4,6-Trinitrotoluene (TNT) is an explosive widely used by the military (Gorontzy et al. 1994). Past handling practices at facilities manufacturing, processing, and disposing of explosives have resulted in extensive environmental contamination (Funk et al. 1993; Rieger and Knackmuss 1995). These contaminants have often leached from disposal lagoons into the surrounding soil and groundwater. Lagoon disposal of wastewaters containing explosives is no longer practiced, but thousands of acres of contaminated soil still need to be cleaned up (Funk et al. 1993). Although it is no longer manufactured in the United States, large amounts of TNT-contaminated wastewater continue to be generated at military installations through (1) load, assembly, and packing (LAP), and (2) demilitarization of munitions. These wastewaters vary in the concentration and type of explosives, but frequently contain trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Concurrent Technologies Corporation 1995). TNT is photochemically active, often turning wastewater a pinkish hue, resulting in the generic name "pinkwater" for any wastewater containing TNT. The potential for pinkwater production is large; a survey of four Army installations carrying out LAP operations showed they produce approximately 1.6 million gal (6 million L) of pinkwater annually (Concurrent Technologies Corporation 1995). Granular activated carbon (GAC) adsorption is the standard technology for treating pinkwater and maintaining discharges within limits established under the National Pollutant Discharge Elimination System (Concurrent Technologies Corporation 1995). However, explosives-laden GAC must be regenerated for reuse or destroyed by incineration.

Biological treatment may be an alternative technology for treating TNT-contaminated wastewaters. Because TNT has a high electron deficiency, the initial microbial transformation is reductive (Vorbeck et al. 1998). Under aerobic conditions, a variety of partially reduced TNT daughter products are produced (Vorbeck et al. 1998; Pasti-Grigsby et al. 1996; Fiorella and Spain 1997), resulting in the convergence of TNT to aminodinitrotoluenes,

diaminonitrotoluenes, and/or tetranitroazoxytoluenes (Roberts, Ahmad, and Pendharkar. 1996). Under aerobic conditions, these metabolites are resistant to further degradation and therefore are considered dead-end products (Vorbeck et al. 1998). Anaerobic conditions are required for the complete reduction of TNT to triaminotoluene (Boopathy and Kulpa 1992; McCormick, Feeherry, and Levinson 1976; Preuss, Fimpel, and Diekert 1993; Funk et al. 1993). The biodegradation pathway is thought to proceed through sequential reductions of the *para* and *ortho* nitro groups to 2,4-diamino-6-nitrotoluene, followed by a final reduction of the remaining nitro group to triaminotoluene (TAT) (McCormick, Feeherry, and Levinson 1976; Fiorella and Spain 1997). However, most studies have only observed relatively small concentrations of TAT (McCormick, Feeherry, and Levinson 1976; Funk et al. 1993; Krumholz et al. 1997) or have presumed its formation based on other evidence (Boopathy and Kulpa 1992). Significant amounts of TAT formation from TNT have been demonstrated in research using pure cultures of sulfate-reducing bacteria (Preuss, Fimpel, and Diekert 1993) and fermentative bacteria (Lewis et al. 1996). The fate of TAT is largely unknown, although Lewis et al. identified phenolic products of TAT hydrolysis and an adduct of TAT, apparently formed by the condensation of TAT and pyruvic aldehyde (Lewis et al. 1996).

The susceptibility of nitroaromatic compounds to anaerobic biodegradation prompted a study evaluating a fluidized-bed granular activated carbon bioreactor for treating pinkwater (Concurrent Technologies Corporation 1995). The effluent from the bioreactor was nontoxic and contained no traces of the original components. Although TNT was removed from the wastewater, its fate was unknown. A later study using a conceptually similar two-stage anaerobic-aerobic treatment system evaluated the transformation of TNT (VanderLoop, et al. 1998). The authors concluded that at least 65 percent of the influent nitrogen attributed to TNT is recovered in the final effluent as ammonia, nitrite, nitrate, or biomass. Few TNT reduction products were observed in the effluent from the anaerobic bioreactor. 2,4-Diamino-6-nitrotoluene was the only TNT reduction product identified, and was only observed under nitrogen-limiting conditions. This study forms the basis for a more cost-effective treatment process for Army Ammunition Plants that produce pinkwater.

## Objectives

The objective of this study was to investigate the fate of TNT under methanogenic conditions where CO<sub>2</sub> is the only available electron acceptor.

## Approach

1. A literature review was conducted of laboratory and field studies involving transformation/degradation of TNT by biological treatment.
2. TNT was obtained from Holston Army Ammunition Plant, Kingsport, TN. TAT was obtained from Chem Service, Inc., West Chester, PA.
3. Biodegradation studies were carried out in serum bottles using sludge and wastewater obtained from a municipal and industrial wastewater treatment plant, respectively.
4. TNT and biodegradation intermediates were taken periodically from the serum bottles and analyzed by high pressure liquid chromatography or capillary electrophoresis.
5. The results were analyzed and conclusions were drawn based on the results of the analyses.

## Scope

This work reports on the degradation of TNT under methanogenic conditions, demonstrating, for the first time, near stoichiometric formation of TAT from TNT.

## Mode of Technology Transfer

It is anticipated that the information derived from this basic research will form the basis for further research for developing and implementing anaerobic biological treatment systems for treating explosive contaminated material wastewaters.

This report is available on the CERL web page at <http://www.cecer.army.mil>.

## 2 Materials and Methods

### Chemicals

TNT was obtained from Holston Army Ammunition Plant (AAP) in Kingsport, TN. 2,4,6-triaminotoluene trihydrochloride (TAT) was obtained from Chem Service, Inc., West Chester, PA. 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 2,4-diamino-6-nitrotoluene (24DA6NT), and 2,6-diamino-4-nitrotoluene (26DA4NT) were obtained from AccuStandard, Inc., New Haven, CT, and were 95 percent purity or greater.

### Source of Inoculum for Biodegradation Studies

Biodegradation studies were carried out in serum bottles using sludge obtained from industrial and municipal wastewater treatment plants. The industrial wastewater treatment plant was located at the Holston AAP in Kingsport, TN. The treatment plant receives wastewater contaminated with RDX and HMX. Wastewater samples were collected from the beginning segment of the treatment plant (known as the anoxic filter) and were stored at 4°C until use. Anaerobic digester sludge from the municipal wastewater treatment plant for the city of Urbana, IL, was also used.

### Serum Bottle Biodegradation Studies

Biodegradation of TNT was evaluated by comparing substrate disappearance in experimental bottles to that in sterile controls. TNT was added to serum bottles (160 ml total volume) containing 100 ml of a basal salts medium amended with 10 to 20 percent sludge (vol/vol). Sterile basal salts medium (steam sterilization, 121°C, 20 min) containing resazurin (0.0002 percent) was added to the serum bottles, followed by the addition of sludge. The basal salts medium consisted of the following per liter; NaCl, 0.8 g;  $\text{NH}_4\text{Cl}$ , 1.0 g; KCl, 0.1 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g;  $\text{KH}_2\text{PO}_4$ , 1.35;  $\text{K}_2\text{HPO}_4$ , 1.75 g;  $\text{NaHCO}_3$ , 1.0 g; trace metal solution, 10 ml; vitamins, 10 ml. Trace metal and vitamin solutions were made as described by

Tanner, McInerney, and Nagle (1989). The pH of the medium was adjusted to 7.2. The medium was prepared and dispensed using strict anoxic techniques as described by Shelton and Tiedje (1984). After addition of the sludge, the bottles were sealed with black butyl rubber stoppers and aluminum crimp seals. The headspace of the bottles was evacuated and replaced with a mixture of  $N_2:CO_2$  (80:20) three times and then pressurized to 1.3 ATM. Sterile controls were prepared by adding  $HgCl_2$  (5 mM final concentration) and autoclaving three times on three subsequent days (steam sterilization, 121 °C, 20 minutes). A stock solution of TNT (1,100  $\mu M$ ) was made in deionized water and added to the serum bottles to target concentrations ranging from 50 to 200  $\mu M$ . The study was conducted in triplicate in the dark at room temperature.

## Analytical Methods

TNT, 2A46DNT, 4A26DNT, 24DA6NT, and 26DA4NT were analyzed by reverse phase HPLC using a Waters LC Module 1 system or by capillary electrophoresis as described by Chow et al. (1998).

For HPLC analysis, the following conditions were used initially for analyzing TNT, 2A46DNT, 4A26DNT, 24DA6NT, and 26DA4NT: mobile phase, 60:40 (acetonitrile:50 mM acetate buffer, pH 4.5); injection volume, 20  $\mu l$ ; flow rate, 1.1 ml/minute; wavelength, 254 nm; column, Lichrosphere C-18 reverse phase column (250 mm x 4.6 mm, 5  $\mu m$  particles; Alltech Associates, Inc., Deerfield, IL). Because 2A46DNT and 4A26DNT co-eluted using this mobile phase, we were unable to quantitate these compounds.

Later analyses used a 50 percent methanol:50 percent acetate buffer (50 mM, pH 4.5) mobile phase with the other parameters identical to those listed above. This mobile phase resulted in the separation and quantitation of all TNT reduction products including 2A46DNT and 4A26DNT except triaminotoluene. Identification of unknown compounds was by comparison of their retention time with those of standards.

Triaminotoluene was analyzed using a Waters HPLC model 600E liquid chromatograph equipped with a 717 Plus autosampler and 991 photodiode array (PDA) detector. PDA data was collected from 200-310 nm, and extracted chromatograms were reproduced at 215 nm. HPLC analyses were performed with a Supelcosil LC-CN column (250 x 4.6 mm, 5  $\mu m$  particles; Supelco, Bellefonte, PA) with a guard column of the same type. An HPLC gradient method retained triaminotoluene on the column in the beginning of the run, but

eluted off the TNT reduction products toward the end of the run when acetonitrile made up a much higher portion of the mobile phase composition. The mobile phase consisted initially of 99 percent of 10 mM dibasic sodium phosphate buffer (pH 3.2, Aldrich Chemical Co.) and 1 percent of acetonitrile. The flow rate was 1.0 ml/min.

The following gradient mobile phase was used; the total elapsed time after each step gradient is given in parentheses. The initial conditions were 1 percent acetonitrile:99 percent phosphate buffer. After 5 minutes, acetonitrile was increased to 100 percent over a period of 1.5 minutes (6.5 min), maintained for 2 minutes (8.5 min), then decreased to 1 percent acetonitrile over 4.5 minutes (13 min) and maintained for 7 minutes (20 min). The total elapsed time between sample injections was 20 min.

The headspace of the serum bottles was monitored for the formation of  $\text{CH}_4$  by gas chromatography. Gas samples were injected into a gas chromatograph (GC) equipped with a flame ionization detector and a Porapak-Q packed column (Alltech Associates, Inc., Deerfield, IL). The GC conditions were: helium flow rate, 30 ml/min; injector, oven, and detector temperatures, 75°C.

## Hydrolysis Procedure

Transformation of TNT typically results in the formation of reduced TNT products, such as aminodinitrotoluenes and diaminonitrotoluenes. The reduction of the nitro group proceeds through the nitroso and hydroxylamino compounds (McCormick et al 1976). These intermediates are reactive and will bind to organic matter. The hydrolysis procedure, used for releasing TNT bound to organic matter, was carried out by a procedure developed by Thorne and Leggett (1997).

## Sampling

Liquid samples were taken periodically from the serum bottles using a syringe and needle. Sample preparation for capillary electrophoresis consisted of centrifuging the samples at 12,000 x g for 4 minutes, followed by filtration (0.2  $\mu\text{m}$ ). Samples for HPLC analysis were mixed 1:1 with acetonitrile and stored at -20 °C until testing. After thawing, the samples were centrifuged (12,000 x g, 4 minutes). Due to the susceptibility of TAT to hydrolysis and abiotic degradation, samples were prepared as above, but run the same day they were taken.

Methane concentrations were determined by taking samples of the headspace gas (0.2 ml) using a 1-ml disposable syringe fitted with a 21-ga needle and injecting the sample directly into the GC as described above.

### 3 Results and Discussion

In the initial studies using inoculum from the Holston AAP, approximately 200  $\mu\text{M}$  TNT was rapidly depleted in the serum bottles and was no longer detected after 13 days. While there was no apparent lag in methane production in TNT unamended controls, methane production in TNT amended bottles was inhibited until TNT and the subsequent diaminonitrotoluene isomers had been depleted. Because of the large amount of background methane production, it was difficult to evaluate differences in methane production between the unamended controls and experimental bottles. The bottles were allowed to incubate until methane production had slowed significantly. The headspace of the bottles was flushed and replaced with  $\text{N}_2:\text{CO}_2$  before respiking with 100  $\mu\text{M}$  TNT. TNT was rapidly depleted and not detected after the first day of incubation (Figure 1).<sup>\*</sup> More than 70 percent of the beginning TNT concentration was accounted for as the monoaminodinitrotoluene and diaminonitrotoluene isomers (Figure 1). The *ortho* nitro group of TNT was preferentially reduced relative to the *para* nitro group. For example, 2-amino-4,6-dinitrotoluene was preferentially formed relative to the 4-amino-2,6-dinitrotoluene isomer. Throughout the incubation period, 2A46DNT accounted for more than 80 percent of the two monoaminodinitrotoluene isomers (Figure 1). This is in contrast to reports observing a preferential reduction of TNT at the *para* nitro group (Pasti-Grigsby et al. 1996; McCormick, Feeherry, and Levinson 1976; Fiorella and Spain 1997). Further reduction of the monoaminonitrotoluene isomers led to the formation of 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene (Figure 1). Interestingly, we observed the formation of 2,6-diamino-4-nitrotoluene. Observations of this diaminonitrotoluene isomer are infrequent (Price, Brannon, and Hayes 1995), however, we consistently observed its formation in numerous test runs with sludge obtained from both the municipal and industrial wastewater treatment plants. The diaminonitrotoluenes were not persistent. Their fate was unknown, but we suspected triaminotoluene as a likely product based on previous studies demonstrating its formation (Funk et al. 1993; McCormick, Feeherry, and Levinson 1976; and Preuss, Fimpel, and Diekert 1993).

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<sup>\*</sup> Figures and Tables are located at the end of the chapter.



A transitory peak was observed in HPLC chromatograms, which we tentatively identified as triaminotoluene. Later analyses of archived samples by HPLC equipped with a photodiode array detector demonstrated it was not triaminotoluene. Analyses of the serum bottles, however, did demonstrate the presence of triaminotoluene in low concentrations. This trace amount of TAT could not possibly account for the approximately 350  $\mu\text{M}$  TNT added to these bottles. We thought the TNT reduction products may have become bound to the organic matter. The samples were hydrolyzed using an acid-base hydrolysis procedure (Thorne and Leggett 1997). No TNT or other reduction products were observed after hydrolysis (data not shown), suggesting that binding to organic matter was not responsible for their disappearance.

TNT was only partially transformed in sterile controls. At the end of 24 days, all the TNT was accounted for as the following (expressed as a percentage of the initial TNT concentration): TNT, 26 percent; 2A46DNT, 52 percent; 4A26DNT, 14 percent; 24DA6NT, 4 percent; 26DA4NT, 4 percent. The *ortho* nitro group of TNT was preferentially reduced rather than the *para* group, similar to that observed in live incubations. We were slightly surprised at the formation of the diaminonitrotoluene isomers in the sterile controls, but this has been observed by others as well (Krumholz et al. 1997).

Although the fate of TNT was unknown, we suspected the diaminonitrotoluene isomers converged to triaminotoluene, based in part because it was detected at the end of the experiment in low concentrations. TAT is known to be unstable (Preuss, Fimpel, and Diekert 1993; Krumholz et al. 1997; Ederer, Lewis, and Crawford 1997) and its detection can be problematic because of this (Preuss, Fimpel, and Diekert 1993; Krumholz et al. 1997). To determine if TAT was a major intermediate during the anaerobic transformation of TNT, we repeated the experiment, but analyzed for TAT within 8 hours of taking the liquid samples. The biological activity (i.e., methane production rates) in the bottles had decreased substantially; therefore, glucose was added as a cosubstrate to ensure active methane production. Soon after the addition of TNT, a peak appeared in the HPLC chromatogram of the samples with a retention time identical to that of a TAT standard. The spectrum index plot of a TAT standard (Figure 2) was identical to that of the TNT degradation intermediate (Figure 3). The purity angle of the peak indicated it was a very pure peak (less than 1) with no other contaminants (Table 1). TAT increased to near stoichiometric concentrations (Figure 4). After 10 days of incubation, nearly 75 percent of the TNT could be accounted for as TAT. As TAT concentrations were decreasing near the end of the incubation, the spectrum index plot looked less like the authentic TAT standard (Figures 5 and 6). The purity angle also increased, indicating other

compounds were coeluting with the TAT (Table 1). This suggested that TAT was being degraded further. No triaminotoluene was observed in cosubstrate unamended controls or sterile controls (data not shown).

The addition of TNT to the serum bottles was inhibitory to methane formation (Figure 7). Before respiking with TNT, methane production rates in the experimental bottles and unamended controls were similar. Methane formation stopped immediately upon respiking with TNT, while there was no decrease in methane production rates in the TNT unamended controls (Figure 7). In the bottles respiked with TNT, methane production resumed when the newly formed diaminonitrotoluenes were almost depleted. After 60 days of incubation, methane production in bottles amended with TNT was less than the unamended controls, suggesting there was no conversion of the ring carbons to  $\text{CH}_4$  and  $\text{CO}_2$ . The inhibition of methane production by TNT was not surprising since nitroaromatic compounds are known to lyse methanogenic bacteria and inhibit methane formation in anaerobic sewage sludge (Gorontzy, Kuver, and Blotevogel 1993). TNT is also known to be mutagenic and toxic to microorganisms (Roberts, Ahmad, and Pendharkar 1996). We observed a similar phenomena with a RDX-degrading methanogenic enrichment culture growing on ethanol (Adrian and Sutherland, DRAFT). Methane production was inhibited upon the addition of RDX, but resumed when RDX was no longer detected in the liquid phase. The mechanism of methane inhibition appears to involve the competition of RDX-degrading microorganisms and methanogens for  $\text{H}_2$  produced during the metabolism of ethanol. There is only one other report we are aware of suggesting such a phenomenon. Boopathy and Kulpa reported TNT may substitute for sulfate as catabolic electron acceptors for *Desulfovibrio spp.* (Boopathy and Kulpa 1992).

Evaluation of the effect of substrates on TNT degradation showed that some cosubstrates enhanced TNT degradation rates relative to unamended controls (Table 2). For example, the addition of glucose resulted in a TNT degradation rate of  $6.3 \mu\text{M day}^{-1}$ , the highest rate observed (Table 2). Ethanol also enhanced the TNT degradation rate, resulting in a degradation rate of  $4.2 \mu\text{M day}^{-1}$ , a 1.9-fold increase over the cosubstrate unamended control. TNT degradation rates in bottles amended with acetate were similar to those in the unamended control, indicating acetate had no effect (Table 2). The influence of cosubstrates on degradation rates may be related to their ability to serve as  $\text{H}_2$  donors. Complex substrates are fermented by other members of a bacterial consortium, producing  $\text{H}_2$  as one of the products, which becomes available to the TNT degrading bacteria. TNT degradation rates are enhanced by the addition of cosubstrates, such as glucose and ethanol, which result in the production of  $\text{H}_2$  (Gibson and

Sewell 1992; Gottschalk 1986). The metabolism of acetate does not result in the production of  $H_2$ , therefore it does not enhance TNT degradation rates. Other researchers have reported this also, suggesting that  $H_2$  was the electron donor responsible for stimulating reductive dechlorination reactions (Gibson and Sewell 1992; Fennel and Gossett 1997).

These studies did not give any insight into the subsequent reduction of 2A46DNT. Reduction of the second nitro group could occur either at the *ortho* or *para*-nitro group, resulting in the formation of either 26DA4NT or 24DA6NT, respectively. To determine the fate of 2A46DNT, we used it to spike bottles. In glucose unamended bottles, 2A46DNT was reduced at both the *ortho* and *para* positions (Table 3). After 3 days, 94 percent of the starting substrate was recovered as a mixture of 2A46DNT, 24DA6NT, and 26DA4NT. 26DA4NT accounted for more than 65 percent of the two diaminonitrotoluene isomers, indicating the *ortho* nitro group was preferentially reduced. Interestingly, in glucose amended bottles, 24DA6NT accounted for more than 70 percent of the two diaminonitrotoluene isomers, indicating the *para* nitro group was preferentially reduced, a finding opposite that observed with the glucose unamended bottles (Table 3). In the sterile control, 100 percent of the starting material was recovered as a mixture of 2A46DNT, 24DA6NT, and 26DA4NT (Table 3). The DANT isomers were formed in almost identical amounts. The literature search preliminary to this study revealed no other reports demonstrating the formation of both diaminonitrotoluene isomers from the subsequent reduction of 2A46DNT.

TNT biodegradation proceeded in a stepwise reduction of the nitro groups to triaminotoluene (Figure 8). This biodegradation pathway has several notable differences from pathways published by other researchers. For example, we observed the formation of the 2,6-diamino-4-nitrotoluene isomer, a reduction product not typically reported by others (McCormick, Feeherry, and Levinson 1976; Krumholtz et al. 1997; Preuss, Fimpel, and Diekert 1993; Funk et al. 1993). We are aware of only one other report that noted small amounts of 26DA4NT being formed (Price, Brannon, and Hayes 1995). We also observed the formation of both diaminonitrotoluene isomers before complete reduction of the monoaminonitrotoluene isomers had occurred. Some studies have suggested the aminonitrotoluene must be completely reduced before reduction of the second nitro group occurs (Fiorella and Spain 1997; Haidour and Ramos 1996).

## 4 Conclusions

These studies demonstrated the complete reduction of TNT to a mixture of reduction products that converged to triaminotoluene. The *ortho* nitro group was preferentially reduced, forming 2-amino-4,6-dinitrotoluene, accounting for more than 80 percent of the two monoaminodinitrotoluene isomers. 2-Amino-4,6-dinitrotoluene was further reduced at both the *ortho* and *para* position, forming 2,6-diamino-4-nitrotoluene and 2,4-diamino-6-nitrotoluene, respectively. The reduction products converged to triaminotoluene, accounting for nearly 75 percent of the initial TNT concentration, before being further degraded to unknown products.

These findings suggest that TNT was not mineralized to  $\text{CH}_4$  and  $\text{CO}_2$ , nor bound to the organic matter. Glucose and ethanol enhanced the TNT degradation rate, while acetate had little effect. The influence of cosubstrates on the degradation rates may be related to the cosubstrates' ability to serve as  $\text{H}_2$  donors. Glucose and ethanol are fermented by bacteria, producing  $\text{H}_2$ , which becomes available to the TNT degrading bacteria. The metabolism of acetate does not result in the production of  $\text{H}_2$ ; therefore, it does not enhance TNT degradation rates. This study demonstrates, for the first time, near stoichiometric formation of triaminotoluene from TNT by a mixed culture incubated under methanogenic conditions.

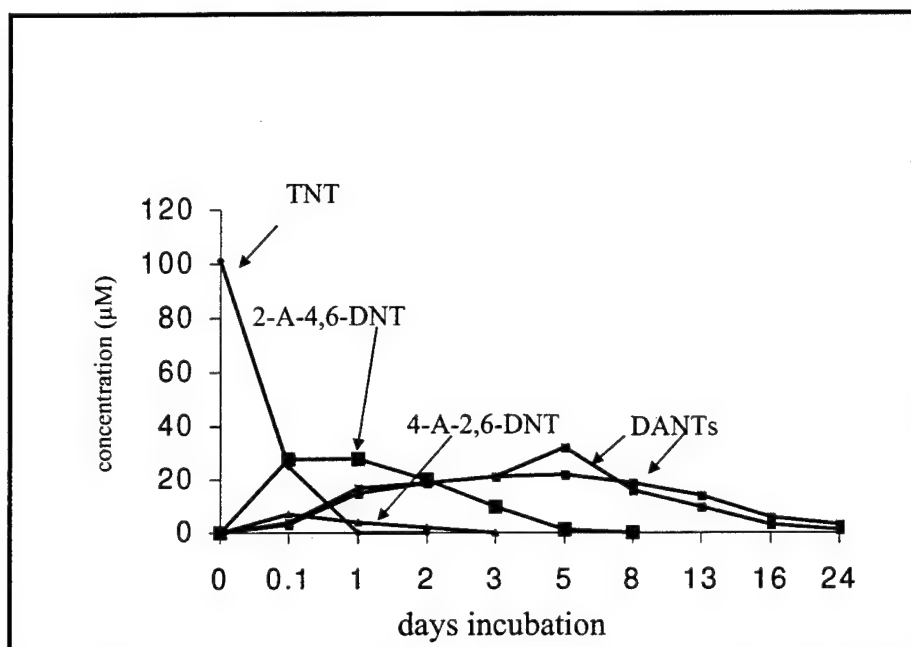


Figure 1. TNT biodegradation in serum bottles incubated under methanogenic conditions.

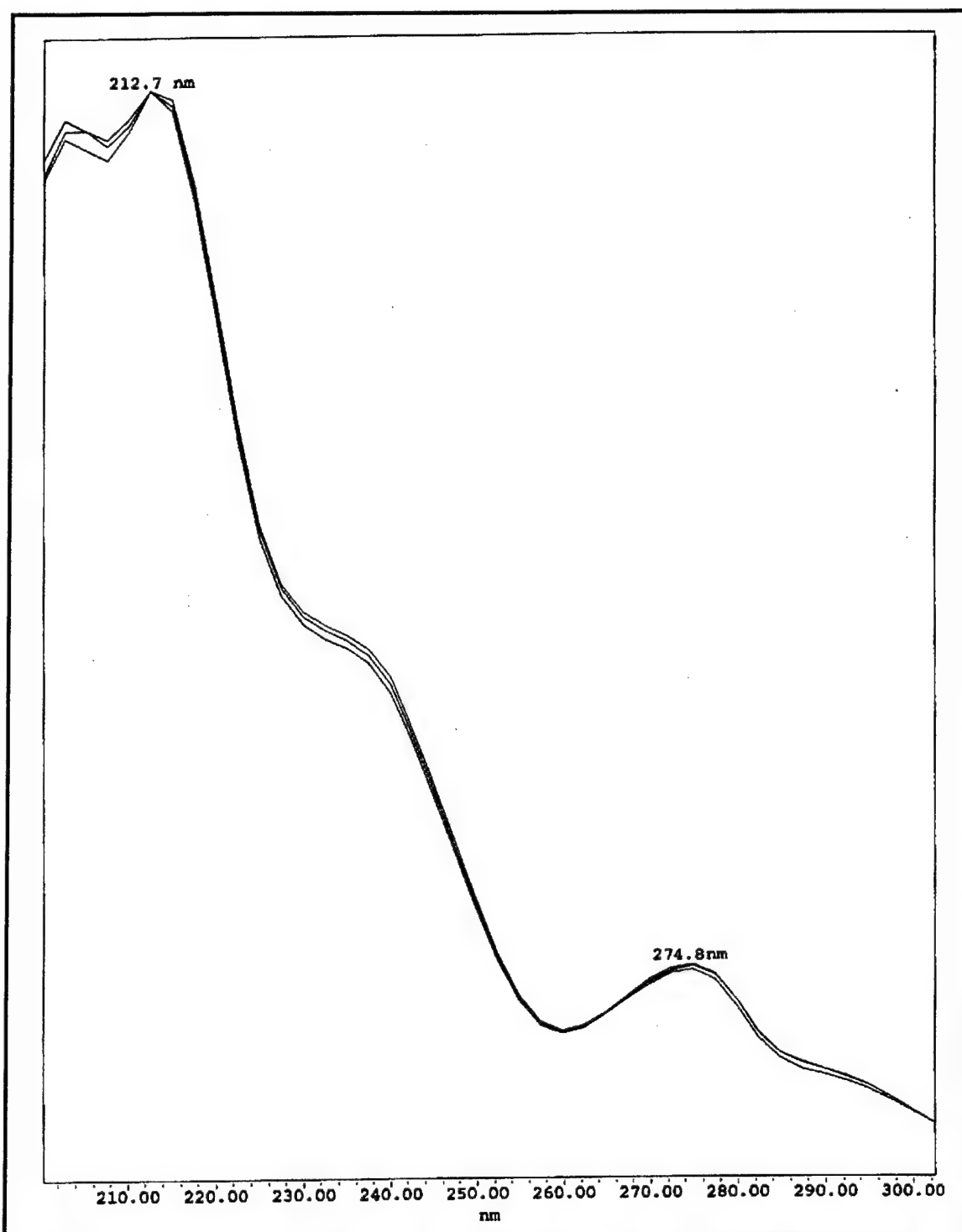


Figure 2. Spectrum index plot of 2,4,6-triaminotoluene standard. The spectrum index is from the apex and two inflection points at half peak height.



Figure 3. Spectrum index plot of TNT biodegradation intermediate after 8 days incubation. The spectrum index is from the apex and two inflection points at half peak height.

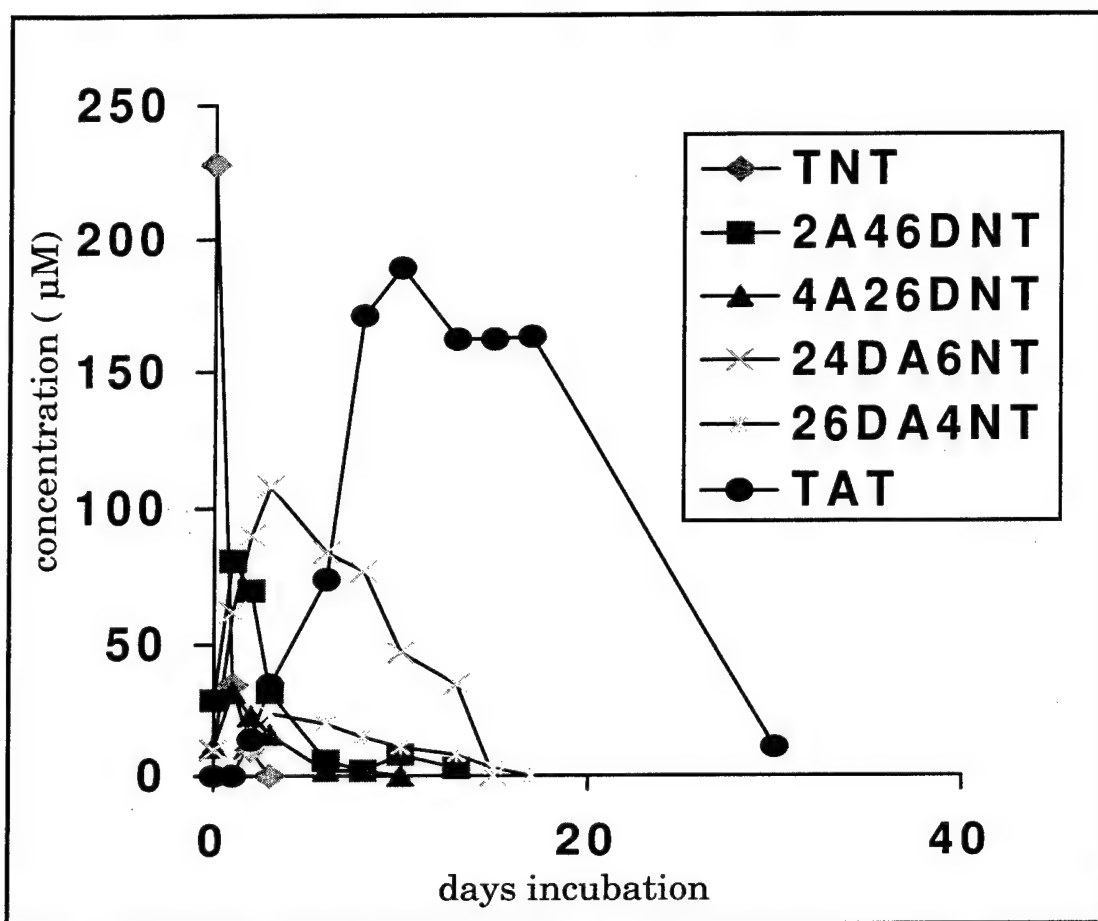


Figure 4. Formation of triaminotoluene during the anaerobic biodegradation of TNT in liquid cultures in serum bottles incubated under methanogenic conditions.



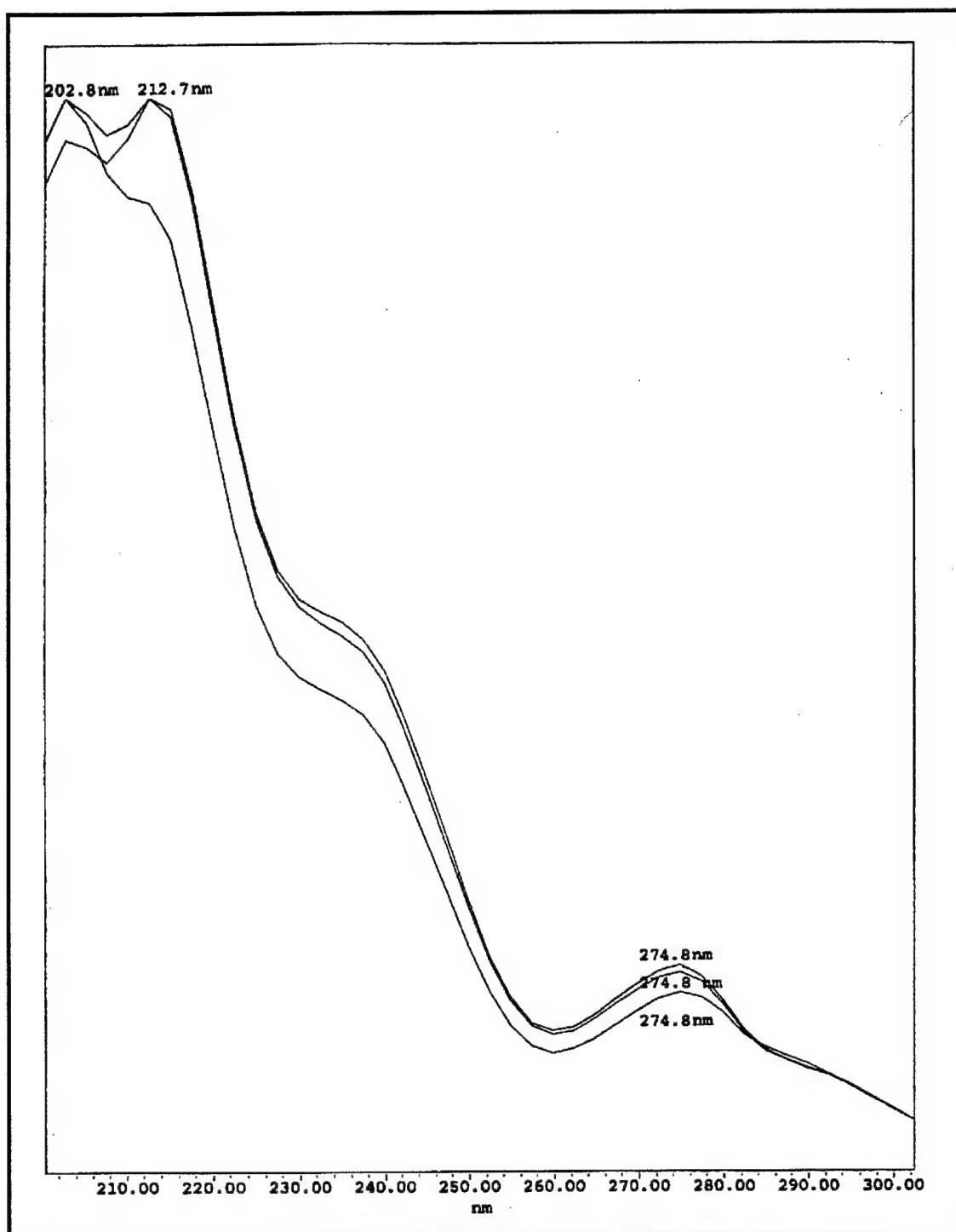


Figure 5. Spectrum index plot of TNT biodegradation intermediate after 17 days incubation. The spectrum index is from the apex and two inflection points at half peak height.

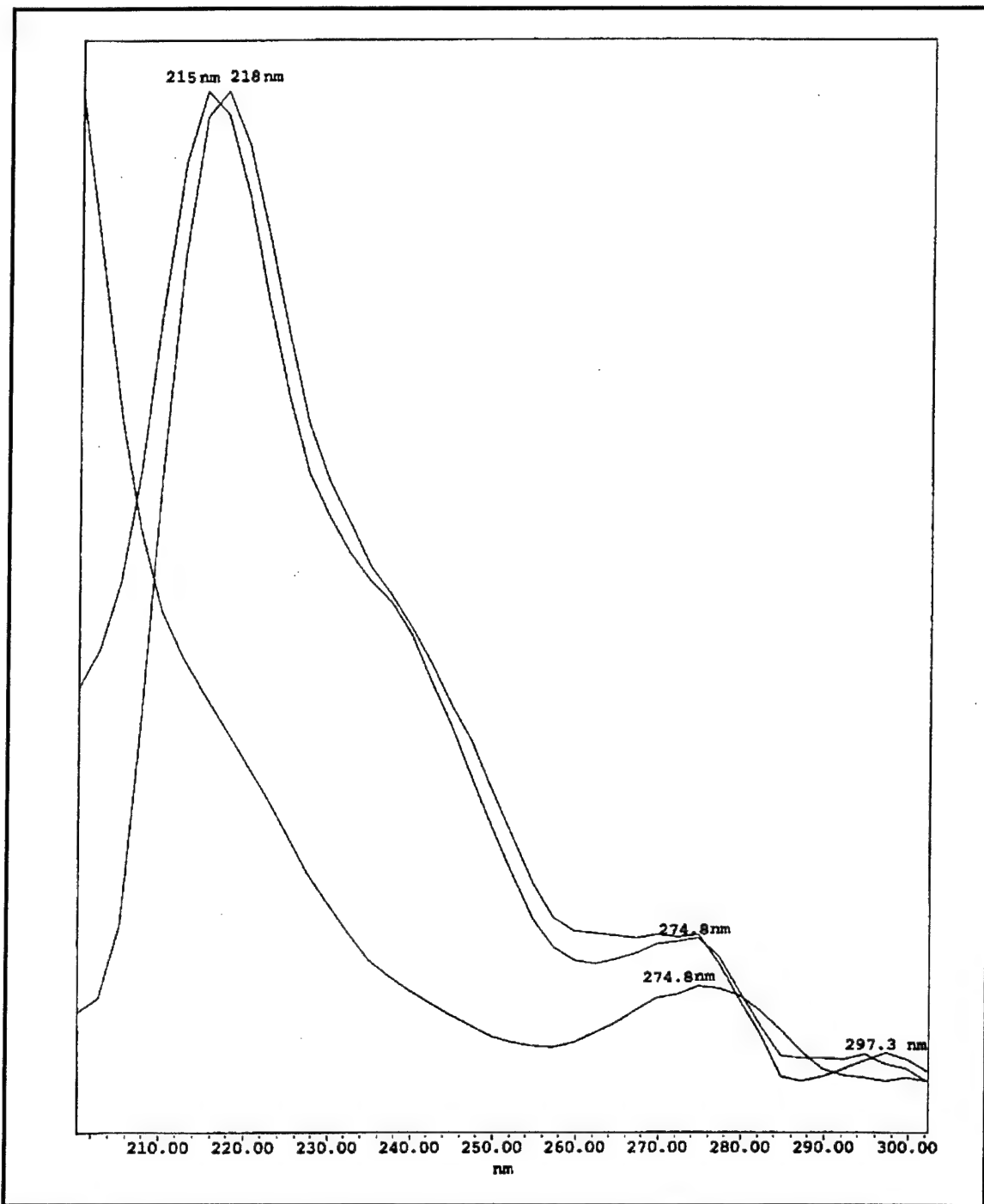


Figure 6. Spectrum index plot of TNT biodegradation intermediate after 30 days incubation. The spectrum index is from the apex and two inflection points at half peak height.

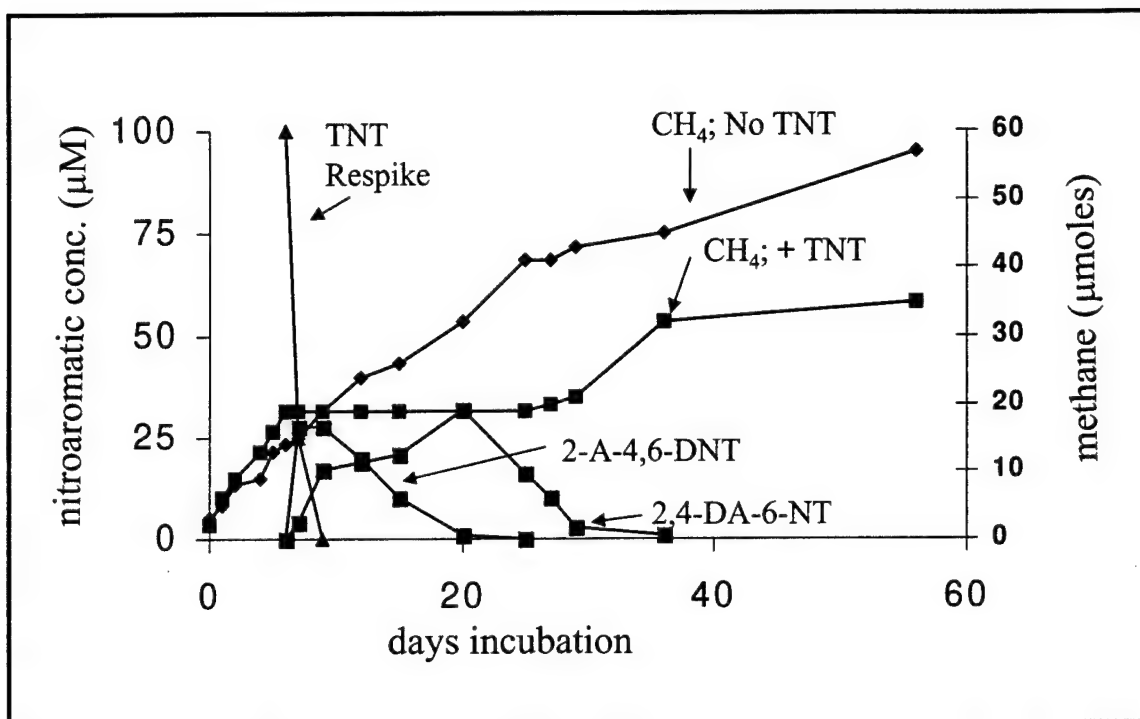
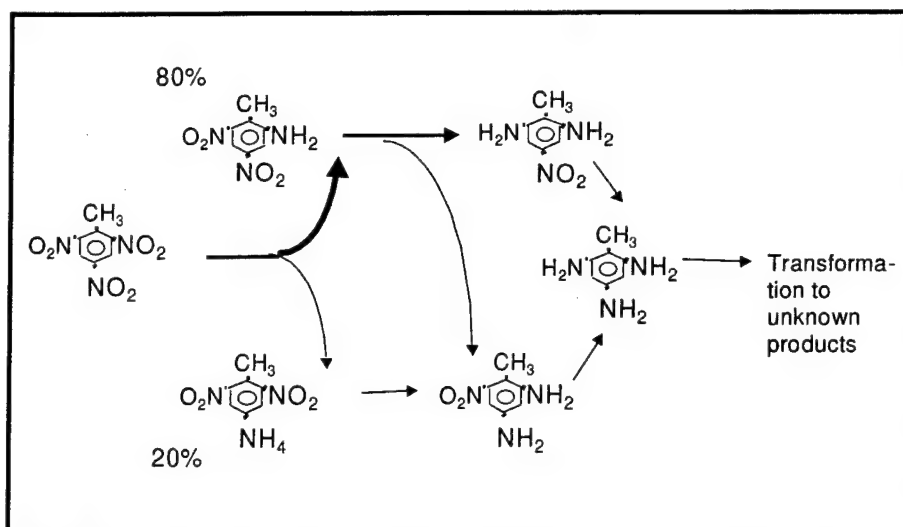


Figure 7. Inhibition of methane production by TNT.



**Figure 8. Anaerobic biodegradation pathway for TNT transformation to triamino-toluene. The relative thickness of the arrows indicate the predominant routes of nitro group reduction observed.**

**Table 1. Purity angles for authentic triaminotoluene and a TNT biodegradation intermediate observed in serum bottles amended with TNT and glucose.**

Triaminotoluene standard	Intermediate, day 8	Intermediate, day 17	Intermediate, day 30
0.49	0.84	2.10	17.4

**Table 2. Degradation rates for TNT in the presence of different cosubstrates.**

Electron Donor	Degradation rate ( $\mu\text{M}$ ) day <sup>-1</sup>
None	2.2
Acetate	2.7
Ethanol	4.2
glucose	6.3

**Table 3. Substrate concentrations (percent) in serum bottles held under methanogenic conditions after spiking with 2A46DNT. The concentrations are normalized to the initial concentration of 2A46DNT added to the serum bottles. The final concentration was determined after 3 days incubation.**

Substrate	Sterile Control		No Cosubstrate		+ glucose	
	Initial	Ending	Initial	Ending	Initial	Ending
2A46DNT	100	70	100	41	100	0
24DA6NT	0	16	0	16	0	30
26DA4NT	0	14	0	29	0	12

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